Guo J, Zhang H, Edwards M, Wang Z, Bai S, He K. Expression patterns of the glycogen phosphorylase gene related to larval diapause in *Ostrinia furnacalis*. *Archives of Insect Biochemistry and Physiology* 2016.

DOI: 10.1002/arch.21318

EXPRESSION OF THE GLYCOGEN PHOSPHORYLASE GENE FROM *Ostrinia furnacalis* (GUENÉE) (LEPIDOPTERA: CRAMBIDAE) IS ACTS IN DIAPAUSE[1]

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Total number of figures: 5

Abbreviated title: Glycogen phosphorylase gene of *Ostrinia furnacalis*

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Abstract: Glycogen phosphorylase is the first enzymatic step in release of glucose from glycogen, a form of energy storage for most organisms. To investigate the characteristics and expression pattern of glycogen phosphorylase gene (*Ofgp*) in Asian corn borer, *Ostrinia furnacalis* (Guenée) larvae, we cloned and analyzed tissue expression of *Ofgp*. The results indicate that the open reading frame (ORF) is 2526 bp, encoding 841aa. The three dimensional structure showed 33 α-helixes and 24 β-sheets. *Ofgp* expression levels varied significantly during the 2^{nd} -5^{th} instars larvae under long day and short day photoperiod; remain low during the pre-diapause phase and then increased after about 36 d under short day photoperiod. In the larvae reared under long day photoperiod, hemolymph ranked the highest in the expression of *Ofgp*. The highest expression was recorded in the fat body and was lower in the other tissues in larvae reared under short day photoperiod. We found that *Ofgp* expression increased linearly from October 2012 to January 2013. The expression was negatively correlated with environmental temperature. We infer the higher *Ofgp* expression may enhance the cold hardiness of the diapause larvae.

Key words: *Ostrinia furnacalis*; glycogen phosphorylase; diapause; photoperiod; temperature; cold hardiness

INTRODUCTION

Many insect species have evolved diapause adaptations to survive under unfavourable environmental conditions. Diapause is generally divided into pre-diapause, diapause and post-diapause stages, and is induced by environmental factors, particularly photoperiod and temperature (Saunders, 2002). During diapause insects are immobile and draw on lipid and glycogen energy stores to meet their low-level energy requirements (Hahn and Denlinger, 2007). Glycogen is a carbohydrate reserve for insects during diapause (Hahn and Denlinger, 2011). It is primarily synthesized and stored in fat bodies where it can be mobilized to fulfill two majors roles during diapause. In one it is an energy source that can be converted to glucose or trehalose to support basal metabolism; in the other, it is converted into sugar-based cryoprotectant molecules such as glycerol and sorbitol (Hahn and Denlinger, 2011). In both situations, mobilization of glycogen reserves begins with the action of glycogen phosphorylase (GP).

The first report of a complete insect glycogen phosphorylase (GP) mRNA sequence came from work on *Drosophila Melanogaster* (Tick et al., 1999). Typically GP is a dimer composed of two identical subunits (Browner and Fletterick, 1992) including a catalytic site, a glycogen binding site, an allosteric site and a Ser-14 residue available for reversible phosphorylation.

Many insect pest species, including Asian corn borer, *O. furnacalis,* express facultative or obligatory diapause. Diapause is a kind of adaptability that contributes to the

abundance of insects and the long-term success of the pest species, which drives considerable interest in insect diapause study. The relevance of diapause and GP has been studied in some insects such as *Bombyx mori* (Yamashita and Hasegawa, 1975; Pant and Jaiswal, 1982; Chandrashekar and Bali, 1987; Ponnuvel et al., 2010), *Pyrrhocoris apterus* (Košťál et al., 2003; 2004) and *Chilo suppressalis* (Li et al., 2002). Extending current knowledge on GP, we cloned and characterized a gene encoding GP from *O. furnacalis* and explored the gene expression under long and short day conditions in this paper.

MATERIAL AND METHODS

Experimental insects

O. furnacalis eggs used in the experiment were a lab strain from the Corn Insect Pest Research Laboratory, Institute of Plant Protection (IPP), Chinese Academy of Agricultural Sciences (CAAS), Beijing, China. The original insects were collected from a corn field near Gongzhuling city, Jilin province (44° N, 125° E) and reared in laboratory for more than ten generations (28 ℃, L16:D8 photoperiod, relative humidity 70-80%). All larvae were reared in constant temperature incubator by agar artificial diet (main ingredients are corn flour, soy flour, yeast, agar and sugar) (Zhou et al., 1980).

First instar larvae maintained under short day and low temperature grow slowly with high mortality and develop irregularly.We maintained 1st larvae (first three days after egg incubation) under long day condition (28 ℃ under L16:D8 photoperiod,

relative humidity 70-80%). From 2nd instar, two treatments were used, a long day treatment as above, and a short day condition (24.5 ℃ under L11:D13 photoperiod, relative humidity 70-80%), the *O. furnacalis* larvae would have a higher diapause rate with the photoperiod L11:D13 (Guo et al., 2013). The larvae were collected 5 d, 8 d, 11 d, 14 d (2nd, 3rd, 4th, 5th instars) and 17 d (pupae) after egg incubation for long day treatment. Due to retarded larval growth under short day condition, larvae for this experiment were taken at 5 d, 9 d, 13 d, 17 d (2nd, 3rd, 4th, 5th instars) and 20 d, 23 d, 26 d, 31 d, 36 d, 41 d after egg incubation.

The newly hatched larvae were released twice into summer corn in the Langfang experimental farm, IPP, CAAS on 15th and 28th in August 2012 to ensure that we can collect enough overwintering larvae. The larvae were collected from corn field once a month from October 2012 to February 2013.

Isolation of total RNA and synthesis of first strand cDNA

Total RNA was isolated using Trizol reagent (Invitrogen Life Technologies, USA). The concentration and purity were assessed with a Nanodrop 2000/2000C instrument. First Strand cDNA Synthesis Kit (Promega, USA) was used for synthesis of the first strand cDNA from 2.0 μg total RNA and reactions were carried out in a total volume of 20 μL as per the instructions. The product was stored at -20℃ for further use.

Cloning of *Ofgp* gene

Primers G-f and G-r (Table 1) used in PCR for clonging the middle fragment were designed after alignment of two GP genes derived from *B. mori* and *Spodoptera exigua*

109 (accession number: EU527367 and FJ754277). All the gene-specific primers were
110 designed utilizing Primer Premier 5.0 (http://www.premierbiosoft.com/). PCR for
111 middle fragment was carried out under the following conditions: an initial denaturation
112 at 94 ℃ for 2.5 min, followed by 30 cycles of 94 ℃ for 30 s, 50 ℃ for 45 s, and
113 72 ℃ for 50 s, and a final extension at 72 ℃ for 10 min. The PCR products was
114 excised and purified using the Gel ExtractionMini Kit (Sangon, China) and cloned into
115 pGEM-T Easy cloning vector (Promega, USA), then transformed to DH5α and cultured
116 on solid LB medium for 16h at 37 ℃. The positive clones were grown in LB^{Amp} broth
117 16h at 37 ℃ before isolation of the recombinant plasmids for sequencing across the
118 cloned insert.
119 Rapid-amplification of cDNA ends (RACE) (Invitrogen, USA) was used to
120 generate full length clones for *Ofgp*. The middle fragment was used as a probe together
121 with the primers 3G, 5G-out, 5G- in, AUAP (in the kit) and G5-X, G5-S (Cavener,
122 1987; Tick et al., 1999) (Table1). The reverse transcription and PCR were carried out
123 as described in the manufacturer's instructions and the *Ofgp* ORF was amplified in its
124 entirety using the full-length primers OfGP-f and OfGP-r.
125 Sequence and structural analysis was performed on the sequenced clones using a
126 variety of bioinformatics tools. Correlated sequences downloaded from NCBI
127 (http://www.ncbi.nlm.nih.gov/) were the source for multiple sequence alignment by
128 DNAMAN. Sequence homology was analysed via BLAST
129 (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and MEGA 4 was used for the phylogenetic

tree construction. The three dimensional structure of OfGP was predicted by Swiss-PdbViewer.

Quantitative real time PCR

Quantification *of Ofgp* mRNA levels was assayed using qPCR utilizing a TaqMan assay method. We used the *β-actin* of *O. furnacalis* as the reference gene (Cui et al., 2013; Xu et al., 2014) and designed primers OfGP-F, OfGP-R, β-actin-F, β-actin-R and probes OfGP-probe, β-actin-probe via Primer Express 3.0. Standard curves of cDNA were generated to evaluate the probes (Fig. S3). qPCR was carried out in 20 μL reactions system containing 1 μL cDNA templates, 10 μL of TaqMan®Universal Master Mix II (ABI, USA), 0.5 μM of forward and reverse primers separately, 0.25 μM of probe and 6.5 μL of ddH_2O. For each sample, reactions were performed in triplicates. Thermal cycling conditions were: 95 ℃ for 8 min, 40 cycles of 95 ℃ for 10 s, 60 ℃ for 20 s.

***Ofgp* expression.**

The total RNA was extracted from 20 *O. furnacalis* larvae for every sample and all experiments for each set of samples were repeated three times. RNA was extracted from the head, gut, hemolymph, fat body, and body wall of 14 d 5^{th} instar larvae under long day condition and 36 d 5^{th} instar larvae under short day condition. RNA was also extracted from the Malpighian tubules under long day conditions. Tissues were collected separately and placed in a 1.5 mL centrifuge tube immediately with liquid nitrogen. 2.0 μg RNA of every sample was used for reverse transcription and the cDNA

were synthesized using random primers. RNA extraction and cDNA synthesis method were the same as described above.

Statistical analysis

The levels of *Ofgp* in 2nd larvae, in body wall and in larvae collected in October 2012 were regarded as references respectively. The $2^{-\Delta\Delta CT}$ method had been used to calculate relative changes in gene expression. The $\Delta C_T = C_{T,\ Target} - C_{T,\ Actin}$; $\Delta\Delta C_T = \Delta C_{T,\ test} - \Delta C_{T,\ control}$ (Livak and Schmittgen, 2001). The significance of difference was compared by single factor analysis of variance and least significant difference (LSD) test was used to determine significant differences at a 95% confidence level ($\alpha = 0.05$). The temperature data from October 2012 to February 2013 was contributed by Professor Yilin Zhou (IPP, CAAS). Multiple comparisons and linear correlation analysis were accomplished using SPSS (SPSS v16.0) software.

RESULTS

Sequence and structure characteristics of *Ofgp*

Amplification revealed a fragment of expected size and subsequent DNA sequencing showed this is the central 685 bp region targeted. The full-length cDNA of *Ofgp* with expected size contained an ORF of 2526 bp nucleotides (Fig S1) encoding 841 amino acids and a 3' untranslated region (3' UTR) of 222 bp. The predicted polypeptide had a putative isoelectric point of 6.04 and molecular mass of 96.49 kDa (GenBank JX113672). The sequence contained the diagnostic residues Ser-14

phosphorylation site, AMP binding site, pyridoxal phosphate cofactor binding site, glucose binding site, glucose-6-P binding site and glycogen storage site that correspond to the active sites residues existed inside the sequence (Fig.S1).Multiple sequence alignment (Fig. 1) showed the N terminal was more conserved. Analysis of the phylogenetic relationships (Fig. 2) constructed with amino acid sequences between 18 glycogen phosphorylase (representing indicated animal classes) showed the closest genetic relationship with *S. exigua*, the other two lepidopterans, *B. mori* and *D. plexippus*, also share high homology with *O. furnacalis*.

The OfGP was symmetrical dimers (Fig. S2F). Each monomer contained 33 α-helixs (Fig. S2A) and 24 β-sheets (Fig. S2B). 3-D imagery indicated that the majority of these were present on the surface of the folded molecule (Fig. S2C) giving *Ofgp* a hydrophilic surface. Some conserved domains as well as regions with high variability found in Fig. 1 were signed in Fig. S2D, which demonstrated the most variant domains were on the surface while conserved regions were inside. Several significant binding sites are shown in Fig. S2F, pyridoxal phosphate (PLP) and glucose binding sites were in the central location of the enzyme.

Ofgp expression in indicated developmental stages

The standard curve slopes of *Ofgp* and *β-actin* (Fig. S3) were -3.332 and -3.200 respectively, r^2 were 0.991 and 0.990 which ensured the reliability of the probes. *Ofgp* expression varied from 2nd instar larvae through to pupae under long day condition (Fig.

192 3) (df=4, 14; F=4.52; P<0.05). It increased from 2nd to 4th instars and declined after the
193 4th. *Ofgp* expressed higher in 3rd and 4th instars compared to 2nd instars and pupae.
194 *Ofgp* expression differed among developmental stages (df=9, 29; F=14.90; P<0.05)
195 (Fig. 3). Expression increased, then declined and remained stable from 20 d to 31 d then
196 increased again from 36 d. The expression quantity from 20 d to 31 d was obviously
197 lower than other time and it climbed rapidly at about 36 d. The same trend was found
198 in the 2nd through 5th instar larvae under long day condition however difference in the
199 expression occurred between long and short day conditions except for the 5th instar
200 (Fig. 3).
201 **_Ofgp_ expression in selected tissues**
202 *Ofgp* expression differred among tissues of 14 d 5th instar larvae under long day
203 condition (df=5, 17; F=70.15; P<0.01) and among tissues from 36 d 5th instar larvae
204 under short day photoperiod (df=4, 14; F=123.88; P<0.01) (Fig. 4). For long day larvae,
205 highest *Ofgp* expression occurred in hemolymph, following by Malpighian tubules,
206 with no differences among head, fat body, and gut, but they were all lower than
207 hemolymph and Malpighian tubule The lowest expression was in the body wall.
208 Nevertheless, *Ofgp* was mainly expressed in the fat body for the short day larvae and
209 the expression was 173.2 times the expression in hemolymph, 213.9 times expression
210 ingut, 851.4 times expression in head and 4,158.5 times the expression in body wall.
211 There was no difference of the expression levels among body wall, head and gut. The

212 expression in fat body under short day condition was higher than that under long day
213 condition and higher in hemlymph under long day condition .

214 ***Ofgp* expression throughout the winter months**

215 *Ofgp* expression changed in overwintering larvae from October 2012 to February 2013
216 (*df*=4, 14; *F*=10.72) (Fig. 5). Expression changes were negatively correlated with the
217 changes of the ambient temperature (r^2= 0.85，*P*=0.026) (Fig. 5). Maximum expression
218 appeared in January at about 19-fold higher, compared to expression in insects collected
219 in October. As the ambient temperatures increased during February *Ofgp* expression
220 declined.

221 # DISCUSSION

222 GP is present in almost all organisms. In concurrence with *S. exigua* (Tang et al.,
223 2012) and *B. mori*, the *O. furnacalis Ofgp* encodes an 841 amino acidpolypeptide,
224 which is slightly shorter compared to the *D. melanogaster* GP, encoding 844 amino
225 acids. The homology between all these GPs is high (Tick et al., 1999). We found 33 α-
226 helixes as reported previously (Hudson et al., 1993) and 24 β-sheets in the secondary
227 structure of OfGP. Most β-sheets are surrounded by α-helixs, which may indicate that
228 β-sheets were more conserved. There is more variation among amino acids located on
229 the surface of the three dimensional structure, possibly to protect the enzyme structure.
230 Glucose concentrations are regulated by homeostatic physiology. The accumulation of
231 glycogen can stimulate GP activity, increasing glucose levels; abundant glucose can
232 inhibit GP activity (Newgard et al., 1989). Photoperiod and temperature influence GP

activity. For example, the GP activity of *Antheraea mylitta* after diapausing for 82 days under the photoperiod of L10:D14 is apparently higher than L14:D10 (Pant et al., 1982). Our study focused on the changes in *Ofgp* expression under different photoperiod and temperature conditions during development and entering diapause. The 3rd and 4th instar larvae may need more OfGP protein to degrade glycogen meet the energy demand of rapid growth under long day condition. The lower expression in pupae under long day condition relates to their reduced metabolic rates, particularly as they approach eclosion. The time between 20 d and 31 d under short daysis likely the pre-diapause phase, after which *Ofgp* expression increased sharply in 36 d, consistent with the rapid mobilization of glycogen as larvae entered the diapause stage (Chandrashekar et al., 1987). We surmise *O. furnacalis* larvae accumulate and store energy phase before the 3rd instar, because the 3rd and 4th instars are developing, and need to mobilize glucose energy metabolism. The 5th instar larvae accumulate glycogen for use in metamorphosis or entering diapause.

GP activity is related to cold resistance in insects (Steele, 1982). Glycerol and sorbitol are important cryoprotectants (Storey et al., 2012) and the accumulation of glycerol occurs through GP activition in overwintering larvae of *Chilo suppressalis* (Li et al., 2002). The rapid activation of GP and decreasing of glycogenleads to increasedpolyhydric alcohols (Yamashita et al., 1975). We found that the expression level of *Ofgp* changed along with the ambient temperature, increasing from October 2012 to January 2013. It is similar to the changing of glycerol content in overwintering

larvae in Japan (Goto et al., 2001). We infer that *Ofgp* expression in overwintering larvae relates to generating glycerol for cold resistance. The higher *Ofgp* expression can enhance the cold hardness of *O. furnacalis* larvae in some extent (Koštál et al., 2004).

Glycogen is produced and stored in fat body, which also mobilizes glucose to suppor cold hardiness (Pant et al., 1982). To some extent carbohydrates are also stored in hemolymph (Williams et al., 1975). We recorded substantial *Ofgp* expression differences in hemolymph and fat body. We assign this difference to differences in the relative amounts if each tissue. Long day larvae were dissected at age 14 d and short day larvae at 36 d. There is much more hemolymph in 14 d larvae compared to 36 d larvae and this is reflected in differences in hemolymph *Ofgp* expresssion.

LITERATURE CITED

Browner MF, Fletterick RJ. 1992. Phosphorylase: a biological transducer. Trends Biochem Sci 17:66-71.

Cavener DR. 1987. Comparison of the consensus sequence flanking translational start sites in *Drosophila* and vertebrates. Nucleic Acids Res 15:1353-1361.

Chandrashekar PM, Bali G. 1987. Glycogen level and glycogen phosphorylase activity in the eggs of silkworm *Bombyx mori* L. Anim Sci 96:49-54.

Cui L, Yang DB, Yan XJ, Rui CH, Wang ZY, Yuan HZ. 2013. Molecular Cloning, Characterization and Expression Profiling of a Ryanodine Receptor Gene in Asian Corn Borer, *Ostrinia furnacalis* (Guenée). PLoS ONE 8: e75825.

Goto M, Sekine Y, Outa H, Hujikura M, Suzuki K. 2001. Relationships between cold hardiness and diapause, and between glycerol and free amino acid contents in overwintering larvae of the oriental corn borer, *Ostrinia furnacalis*. J Insect Physiol 47:157-165.

Guo JQ, Zhang HG, Wang ZY, He KL. 2013. Effects of photoperiod and temperature on diapause induction in *Ostrinia furnacalis* (Lepidoptera: Crambidae)[J]. Acta Entomol Sin 56: 996-1003.

Hahn DA, Denlinger DL. 2011. Energetics of insect diapause. Annu Rev Entomol 56:103-121.

Hahn DA, Denlinger DL. 2007. Meeting the energetic demands of insect diapause: Nutrient storage and utilization. J Insect Physiol 53:760-773.

Hudson JW, Golding GB, Crerar MM. 1993. Evolution of allosteric control in glycogen phosphorylase. J Mol Biol 234:700-721.

Košťál V, Tamura M, Tollarová M, Zahradnickova H. 2004. Enzymatic capacity for accumulation

of polyol cryoprotectants changes during diapause development in the adult red firebug, *Pyrrhocoris apterus*. Physiol Entomol 29:344-355.

Košťál V, Tollarová M, Tamura M, Zahradníčková H, Šula J. 2003. Roles of diapause termination and cold-acclimation in cryoprotectant biosynthesis and accumulation in *Pyrrhocoris apterus* (Heteroptera). International Symposium on Animal and Plant Cold Hardiness 26.

Koštál V, Tollarová M, Šula J. 2004. Adjustments of the enzymatic complement for polyol biosynthesis and accumulation in diapausing cold-acclimated adults of *Pyrrhocoris apterus*. J Insect Physiol 50:303-313.

Li YP, Ding L, Goto M. 2002. Seasonal changes in glycerol content and enzyme activities in overwintering larvae of the Shonai ecotype of the rice stem borer, *Chilo suppressalis* Walker. Arch Insect Biochem 50:53-61.

Li YP, Goto M, Ding L, Tsumuki H. 2002. Diapause development and acclimation regulating enzymes associated with glycerol synthesis in the Shonai ecotype of the ricestem borer larva, *Chilo suppressalis walker*. J Insect Physiol 48:303-310.

Livak KJ, Schmittgen TD. 2001. Analysis of relative gene expression data using Real-Time Quantitative PCR and the 2−ΔΔCT method. Methods 25:402-408.

Mitsumasu K, Kanamori Y, Fujita M, Iwata K, Tanaka D, Kikuta S, Watanabe M, Cornette R, Okuda T, Kikawada T. 2010. Enzymatic control of anhydrobiosis-related accumulation of trehalose in the sleeping chironomid, Polypedilumvanderplanki. FEBS J 277(20):4215-4228.

Newgard CB, Hwang PK, Fletterick RJ. 1989. The family of glycogen phosphorylases: structure and function. Crit Rev Biochem Mol 24:69-99.

309 Pant R, Jaiswal G. 1982. Photoperiodic effect on some enzymes and metabolites in diapausing
310 *Antheraea mylitta* pupae and *Philosamia ricini* larvae during development. J Biosciences
311 4:175-182.
312 Ponnuvel KM, Murthy GN, Awasthi AK, Rao G, Vijayaprakash NB. 2010. Differential gene
313 expression during early embryonic development in diapause and non-diapause eggs of
314 multivoltine silkworm *Bombyx mori*. Indian J Exp Biol 48:1143-1151.
315 Saunders DS. 2002. Insect Clocks. Third ed, Elsevier Science, Amsterdam.
316 Steele JE. 1982. Glycogen phosphorylase in insects. Insect Biochem 12:131-147.
317 Storey KB, Storey JM. 2012. Insect cold hardiness: metabolic, gene, and protein adaptation. Can J
318 Zool 90:456-475.
319 Tang B, Xu Q, Zou Q, Fang Q. 2012. Sequencing and characterization of glycogen synthase and
320 glycogen phosphorylase genes from *Spodoptera exigua* and analysis of their function in
321 starvation and excessive sugar intake. Arch Insect Biochem 80:42-62.
322 Tick G, Cserpan I, Dombradi V, Mechler BM, Torok I, Kiss I. 1999. Structural and functional
323 characterization of the *Drosophila* glycogen phosphorylase gene. Biochem Bioph Res 257:34-
324 43.
325 Williams AJ, Lutz PL. 1975. The role of the haemolymph in the carbohydrate metabolism of
326 *Carcinus maenas*. J Mar Biol Assoc UK 55:667-670.
327 Xu LN, Wang ZY, Zhang J, Ferry N, Edwards MG, Gatehouse AMR, He KL. 2014.
328 Characterization of four midgut aminopeptidase N isozymes from *Ostrinia furnacalis* strains
329 with different susceptibilities to *Bacillus thuringiensis*. J Invertebr Pathol 115: 95-98.

330 Yamashita KS, Hasegawa K. 1975. Glycogen phosphorylase activity in relation to diapause
331 initiation in *Bombyx* eggs. Insect Biochem 5:707-718.
332 Zhou DR, Wang YY, Liu BL, Ju ZL. 1980. Studies on the mass rearing of corn borer: Ⅰ.
333 Development of a satisfactory artificial diet for larval growth. Acta Phytophyl Sin **7**:113-122.
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ACKNOWLEDGEMENTS

We thank Prof. Aocheng Cao from Institute of Plant Protection, Chinese Academy of Agricultural Sciences for offering Quantitative real-time PCR machine and Prof. Yilin Zhou (IPP, CAAS) for offering temperature data in Langfang. Financial support for this study was provided by National 973 Program of the Ministry of Science and Technology of the People's Republic of China (2010CB951503).

342 **Table 1.** Primers used for *Ofgp* cloning and expression analysis.

Primer function	Primer name	Primer sequence（5'-3'）	Annealing tempreture（℃）
Fragment	G-f	TACAACCGCATCAAGCGCGACCC	58
cloning	G-r	CGGTCAGACGAGAACTTGCC	55
5′ RACE	5G-out	CTCCACGCATTGACGAAG	50
	5G-in	GGGTCGTTGTTTACGGTATTGCCCACA	59
3′ RACE	3G	GGCAAGTTCTCGTCTGACCG	55
	AUAP	GGCCACGCGTCGACTAGTAC	56
5′ Amplification	5G-S	CAMMATGAGCGTACAR	38
	5G-X	CAGGCCTCCGTTAC	43
End to end	Gp-f	CACCATGAGCGTACAATCAG	51
	Gp-r	ATACTAGCCAGTTTAGGCGAC	51
Real-time	GP-F	CGTGATCACCCTGTACAACAGAA	54
Quantitative	GP-R	ACAGTCCTCGGCGTGATAGG	55
PCR	GP-probe	(FAM)CAAGCGCGACCCGTCCGC(Eclipse)	58
	β-actin-F	GGCCCAGAGCAAGAGAGGTA	55
	β-actin-R	CGTCCCAGTTGGTGATGATG	53
	actin-probe	(FAM)ACCCTGAAGTACCCCATCGAGCACG (Eclipse)	60

343 FAM is the reporter dye at the 5′end and Eclipse represents the 3′end's quencher dye.

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Figure legends

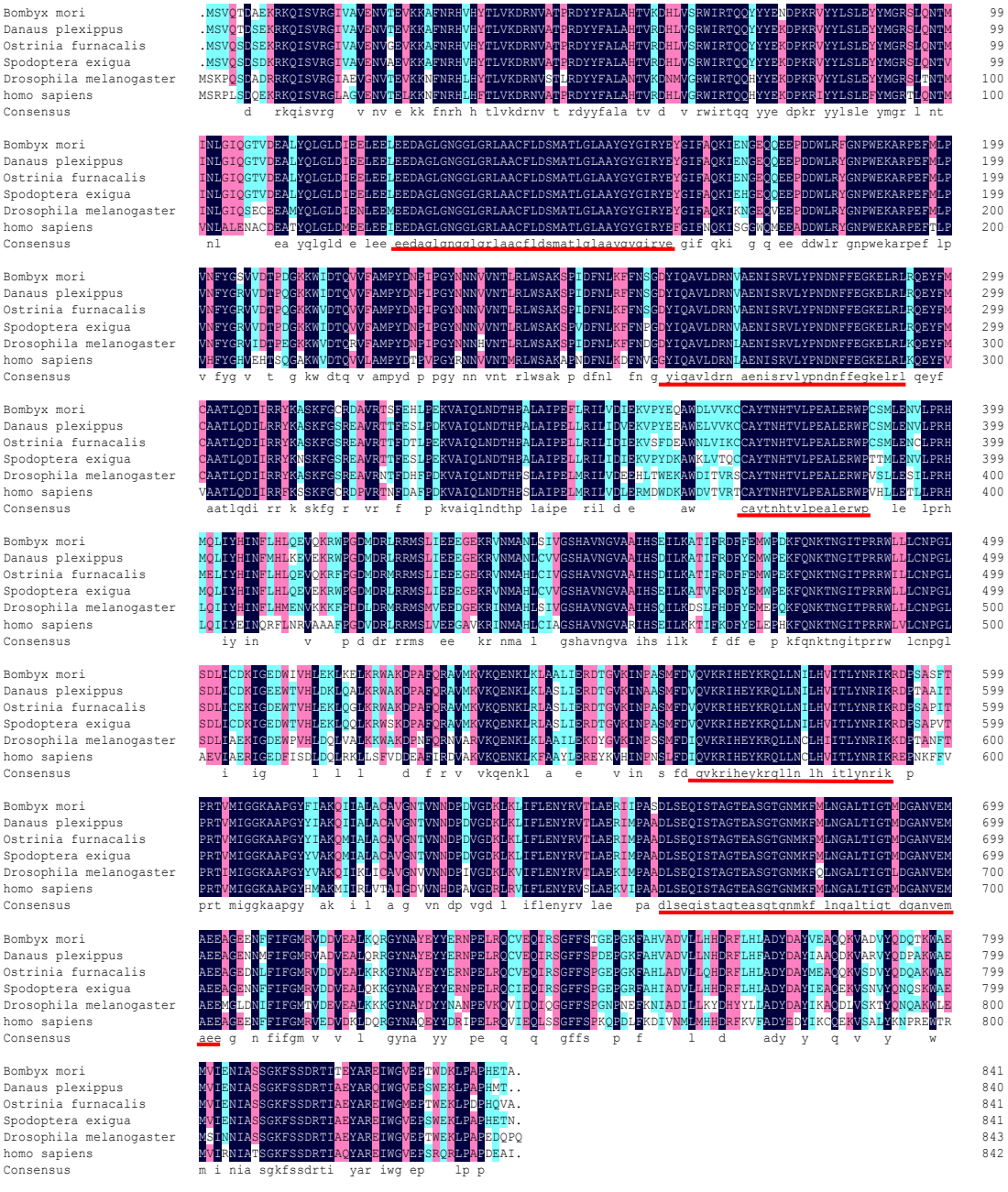
Fig. 1. Multiple sequence alignment of *Ofgp* based on the amino acid sequences. The GenBank accession number for the sequence alignment are: *B. mori* (NP_001116811), *D. plexippus* (EHJ77865), *O. furnacalis* **(**AFO54708), *S. exigua* (ACN78408), *D. melanogaster* (NP_722762), *H. sapiens* muscle form (NP_005600). Sequence fragments labeled with red underline are conserved domains.

Fig. 2. Phylogenetic tree based on amino acid sequences. Full-length amino acid sequences were aligned using the Mega 4.0 program to generate the phylogenetic tree. A bootstrap analysis was carried out and the robustness of each cluster was verified with 1,000 replicates. Values at the cluster branches indicate the results of the bootstrap analysis. The accession number of glycogen phosphorylase sequences are: *O. furnacalis* **(**AFO54708), *S. exigua* (ACN78408), *D. plexippus* (EHJ77865), *B. mori* (NP_001116811), *D. melanogaster* (NP_722762), *A. gambiae* (BAI60046), *A. aegypti* (XP_001650265), *C. quinquefasciatus* (XP_001846386), *A. pisum* (XP_001950760), *A. mellifera* (XP_006561774), *B. impatiens* (XP_003488962), *H. sapiens* liver form (NP_002854), brain form (NP_002853) and muscle form (NP_005600), *P. abelii* muscle form (XP_002821598), *M. musculus* muscle form (NP_035354), *O. aries* muscle form (NP_001009192), *O. cuniculus* muscle form (NP_001075653).

Fig. 3. Relative expression pattern of *Ofgp* in *O. furnacalis* larvae in the indicated developmental stages under long and short day conditions. X-axis is the stage of larvae development, and Y-axis is relative expression of *Ofgp* (mean ±standard error of mean). Different uppercase letters indicate significant difference under long day condition (df=4, 14) at the $P< 0.05$ level and different lowercase letters indicate significant difference under short day condition (df=9, 29) at the $P< 0.05$ level. * means there was significant difference of the expression in the same developmental stage between long day and short day condition at the $P< 0.05$ level.

Fig. 4. Relative expression pattern of *Ofgp* in different tissues of 5th *O. furnacalis* larvae under long day and short day conditions. (The 5th instar larvae of 14 d under long day condition and 36 d under short day condition were used). X-axis is different tissues, and Y-axis is relative expression of *Ofgp* (mean±standard error of mean) showed logarithmically. Different uppercase letters indicate significant difference under long day condition (*df*=5, 17) at the P< 0.01 level and different lowercase letters indicate significant difference under short day condition (*df*=4, 14) at the P< 0.01 level. * means there is significant difference of the expression between long day and short day condition at the P< 0.05 level.

Fig. 5. Relative expression of *Ofgp* in *O. furnacalis* larvae throughout the winter months. X-axis is the month when the larvae were collected, Y-axis on the left is relative expression of *Ofgp* (mean±standard error of mean) and mean monthly temperature on the right which was provided by Prof. Yilin Zhou (IPP, CAAS). Different lowercase letters indicate significant difference among the winter months (*df*=4, 14) at the P< 0.05 level.



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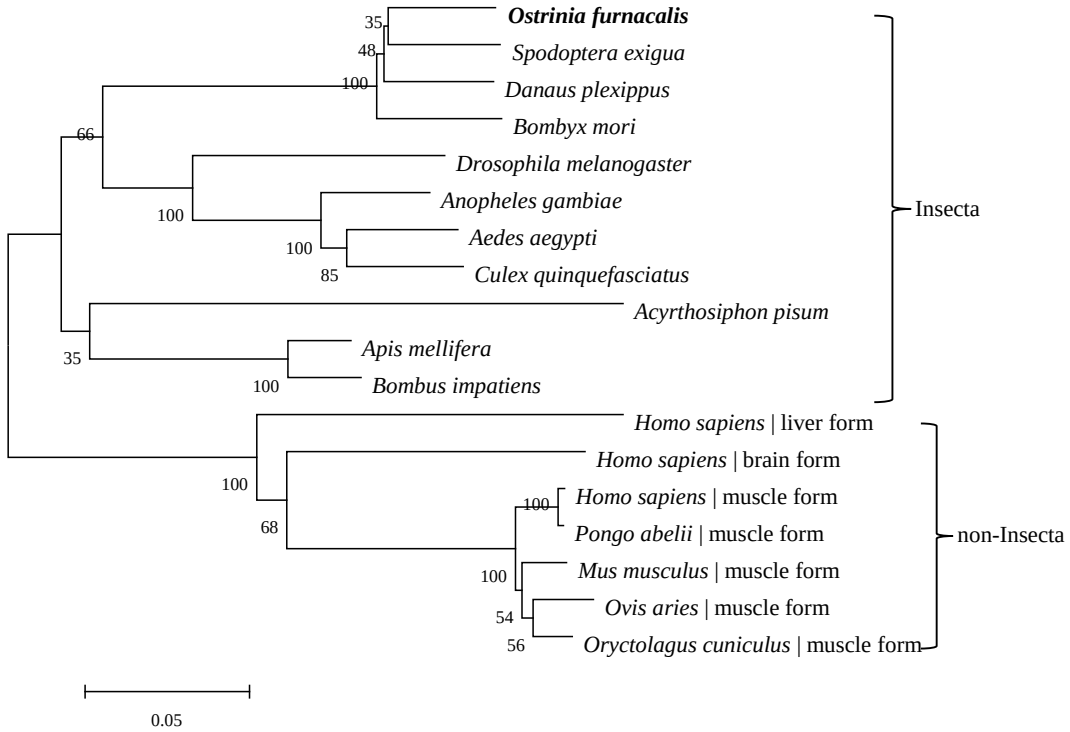

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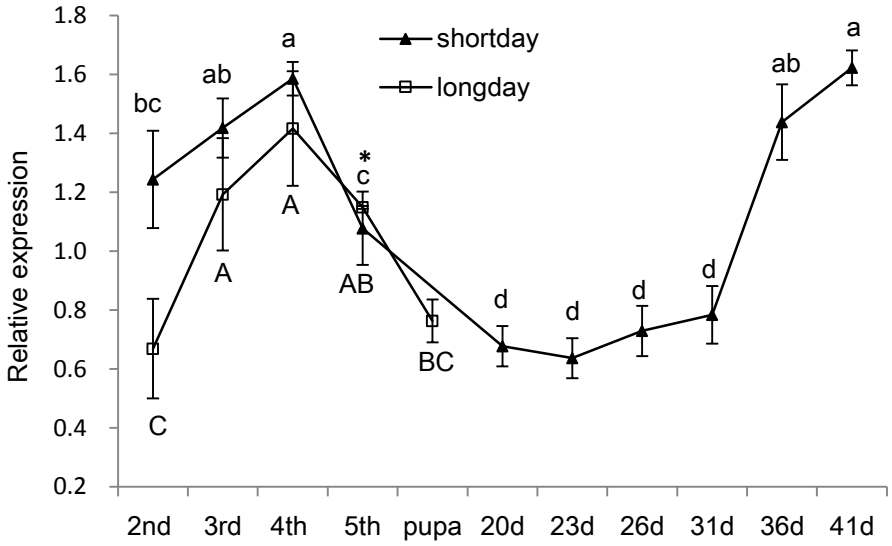

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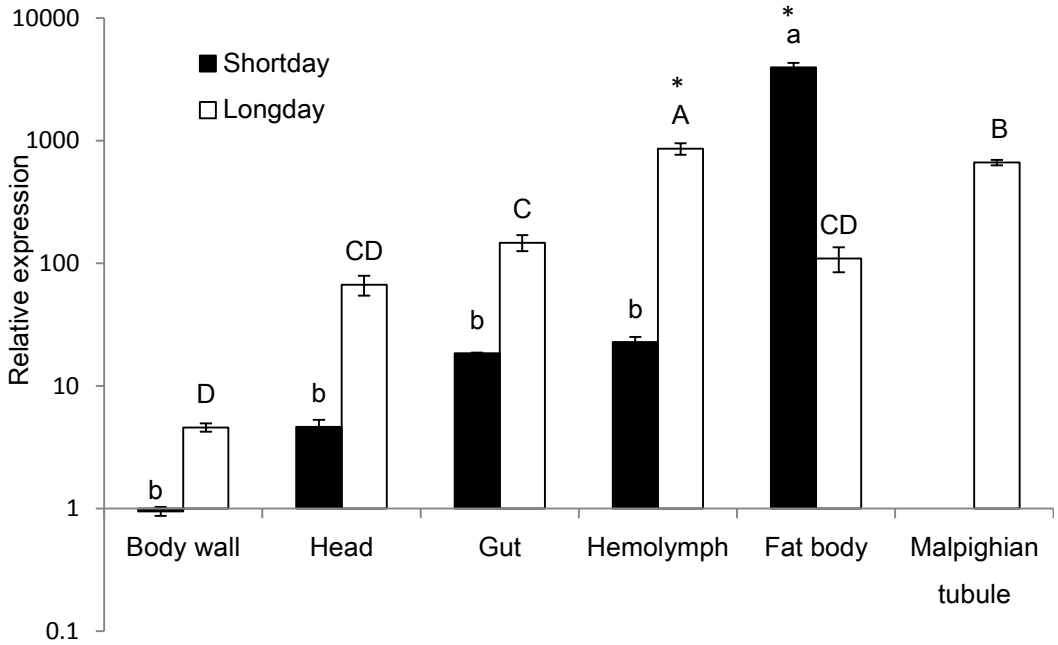

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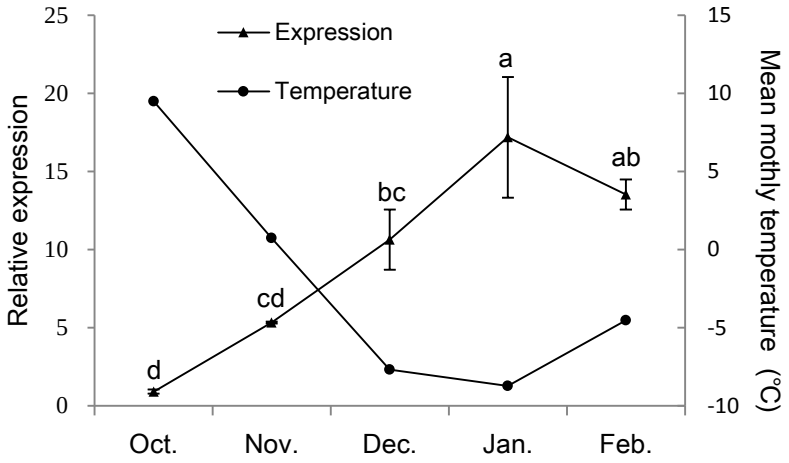

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